RUNX1 mutations in cytogenetically normal acute myeloid leukemia are associated with a poor prognosis and up-regulation of lymphoid genes

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ABSTRACT

Background

The *RUNX1* (*AML1*) gene is a frequent mutational target in myelodysplastic syndromes and acute myeloid leukemia. Previous studies suggested that *RUNX1* mutations may have pathological and prognostic implications.

Design and Methods

We screened 93 patients with cytogenetically normal acute myeloid leukemia for *RUNX1* mutations by capillary sequencing of genomic DNA. Mutation status was then correlated with clinical data and gene expression profiles.

Results

We found that 15 out of 93 (16.1%) patients with cytogenetically normal acute myeloid leukemia had *RUNX1* mutations. Seventy-three patients were enrolled in the AMLCG-99 trial and carried ten *RUNX1* mutations (13.7%). Among these 73 patients *RUNX1* mutations were significantly associated with older age, male sex, absence of *NPM1* mutations and presence of *MLL*-partial tandem duplications. Moreover, *RUNX1*-mutated patients had a lower complete remission rate (30% *versus* 73% P=0.01), lower relapse-free survival rate (3-year relapse-free survival 0% *versus* 30.4%; P=0.002) and lower overall survival rate (3-year overall survival 0% *versus* 34.4%; P<0.001) than patients with wild-type *RUNX1*. *RUNX1* mutations remained associated with shorter overall survival in a multivariate model including age and the European LeukemiaNet acute myeloid leukemia genetic classification as covariates. Patients with *RUNX1* mutations showed a unique gene expression pattern with differential expression of 85 genes. The most prominently up-regulated genes in patients with *RUNX1*-mutated cytogenetically normal acute myeloid leukemia include lymphoid regulators such as HOP homeobox (*HOPX*), deoxynucleotidyltransferase (*DNTT*, terminal) and B-cell linker (*BLNK*), indicating lineage infidelity.

Conclusions

Our findings firmly establish that *RUNX1* mutations are a marker of poor prognosis and provide insights into the pathogenesis of *RUNX1* mutation-positive acute myeloid leukemia. *(ClinicalTrials.gov identifier NCT00266136)*

Key words: *RUNX1*, mutations, prognosis, acute myeloid leukemia.

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The online version of this article has a Supplementary Appendix.

Introduction

The transcription factor *RUNX1* is the fusion partner of *RUNX1T1* (*ETO*) in the recurring t(8;21)(q22;q22) translocation present in 8-13% of adult patients with *de novo* acute myeloid leukemia (AML).[1] *RUNX1* is a key regulator of hematopoiesis and is involved in hematopoietic stem cell emergence and regulation.[2] The structure of the RUNX1 protein is characterized by an N-terminal RUNT domain, which mediates DNA-binding as well as an interaction with core-binding-factor beta (*CBFB*), and a C-terminal transactivation domain.[3] Point mutations in *RUNX1* were initially described in AML secondary to myelodysplastic syndromes, radiation exposure or chemotherapy, with the frequency in these settings being 8 to 10%.[4] Subsequently, analyses of cytogenetically heterogeneous AML cohorts found *RUNX1* mutations in 6-33% of patients.[5-7] The mutational spectrum includes N-terminal missense mutations, affecting mostly the RUNT domain, and C-terminal truncating mutations, deleting the transactivation domain. Both missense and truncating mutations were reported not only to cause a loss of normal *RUNX1* function, but also to act in a dominant negative fashion on the transactivation capacity of wild-type *RUNX1*.[3] In minimally differentiated AML (AML M0) with *RUNX1* mutations, deregulation of lymphoid genes was observed, indicating linage infidelity.[8] Mutations in *RUNX1* are associated with a poor prognosis in cohorts of patients with cytogenetically heterogeneous AML.[5-7] We, therefore, studied the prognostic implications of *RUNX1* mutations in a cohort homogeneous with regards to both cytogenetics (only cytogenetically normal AML; CN-AML) and treatment (all patients treated on the AMLCG-1999 trial). To learn more about the biology of *RUNX1*-mutated AML, we analyzed differential gene expression in cases of CN-AML with *RUNX1* mutations *versus* cases with wild-type *RUNX1*.

Design and Methods

Patients

Ninety-three adult patients with CN-AML with available material and gene expression data were analyzed for *RUNX1* mutations. Seventy-three were enrolled in the multicenter AMLCG-1999 trial of the German AML Cooperative Group, and an additional 20 CN-AML patients were not treated in the trial and could not, therefore, be evaluated for outcome, but were studied for *RUNX1* mutation status and gene expression profiles. Diagnostics were performed centrally at the Laboratory for Leukemia Diagnostics, University of Munich (Germany), and included standard cytomorphology, cytogenetics, fluorescence *in situ* hybridization and testing for *FLT3*-internal tandem duplications (ITD), *MLL*-partial tandem duplications (PTD), and *NPM1*, *CEBPA*, *NRAS*, *KIT*, *IDH1* (R132) and *IDH2* (R140 and R172) mutations. The diagnosis of CN-AML was based on the analysis of at least 20 metaphases in more than 90% of patients, and on the analysis of at least ten metaphases in the remaining patients. All patients received intensive cytarabine-based double-induction and consolidation chemotherapy.[9] The AMLCG-1999 trial is registered at ClinicalTrials.gov (NCT00266136) and was approved by the local institutional review boards of all participating centers. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Mutation screening

The entire open reading frame of *RUNX1* (NM_001754.4) was analyzed from genomic DNA using polymerase chain reaction amplification with exon-spanning primers and bidirectional DNA sequencing on an ABI 3100 Avant instrument. Primer sequences are listed in *Online Supplementary Table S1*.

Microarray analyses

Bone marrow samples taken before treatment was commenced were analyzed using Affymetrix HG-U133 A/B oligonucleotide microarrays (Affymetrix, Santa Clara, CA, USA). Details regarding sample preparation, hybridization and image acquisition have been described previously.[10,11] In order to combine individual oligonucleotide probes to probe sets and to annotate these probe sets to genes, we used custom chip definition files based on the GeneAnnot database (available online at *http://www.xlab.unimo.it/GA_CDF/*).[12] In contrast to standard Affymetrix annotations, in these custom chip definition files each gene is represented by one single probe set comprising only probes that exclusively match the gene of interest. This approach reduces the multiple testing burden by decreasing the total number of probe sets, and potentially increases the specificity of the analyses by eliminating cross-hybridizing probes. Data were normalized using the variance stabilizing normalization algorithm[13] and expression values were calculated by the median polish method.

Differentially expressed probe sets were identified by comparing *RUNX1*-mutated and *RUNX1*-wild type patients, using a permutation-based algorithm to adjust for multiple testing.[14] Genes were called significant if their adjusted q value was <0.05 and the fold change between the two groups was >1.5 or <0.66.[15] Microarray analyses were performed using the R software package, version 2.13.0.[16]

To identify functionally related sets of genes which are deregulated in *RUNX1*-mutated CN-AML, we performed gene set enrichment analysis. Gene sets were obtained from the curated 'canonical pathways' (c2:cp) collection of the Molecular Signatures Database (MSigDB version 3.0.; *http://www.broadinstitute.org/gsea/msigdb/*).[17]

Only gene sets containing between 15 and 200 individual genes (654 of the 880 total gene sets) were included in the analysis. Gene sets were considered significant at a false discovery rate (adjusted for gene set size and multiple testing) of q<0.10

Statistical analysis

Fisher's exact test was used to compare categorical clinical variables of the *RUNX1*-mutated and *RUNX1*-wild-type cohorts. For the continuous variables we used the Mann-Whitney U-test. The clinical endpoints of complete remission, non-responsive AML, relapse-free survival and overall survival were defined as reported previously.[11,18] In brief, patients with more than 5% residual bone marrow blasts after induction treatment were judged to be non-responders. Relapse-free survival was defined as time from the date of complete remission until relapse or death, regardless of cause. Overall survival was defined as time from study entry until death from any cause. Patients alive without an event were censored at the time of their last follow-up.

The prognostic impact of *RUNX1* mutations was first evaluated according to the Kaplan-Meier method and the log-rank test. To adjust for important clinical and molecular prognostic variables, we derived a multivariate Cox model for overall survival with age as a continuous parameter (10-year difference), European LeukemiaNet (ELN) genetic group and *RUNX1* mutational status as covariates.

Results

Patients' characteristics and clinical outcome

In a cohort of 93 adult CN-AML patients, 15 (16.1%) were found to carry *RUNX1* mutations (Figure 1A, Table 1). Four patients carried several *RUNX1* mutations. Among the 73 patients enrolled in the AMLCG-1999 trial, ten (13.7%) had *RUNX1* mutations. The clinical and molecular characteristics of these 73 patients are listed in Table 2. Compared to wild-type *RUNX1*, *RUNX1* mutations were associated with older age (P=0.001), male sex (P=0.005), higher lactate dehydrogenase levels (P=0.003) and a trend towards a lower white blood cell count (P=0.08).

No patient with mutated *RUNX1* carried a concurrent *NPM1* mutation, while the frequency of *NPM1* mutations in the *RUNX1*-wild-type group was 66.7% (P<0.001). *MLL*-PTD were more frequent among patients with *RUNX1*-mutations than among patients with wild-type *RUNX1* (P=0.02). There was no significant association of *RUNX1* mutations with *FLT3*-ITD, *CEBPA*, *NRAS*, *KIT*, *IDH1* (R132), or *IDH2* (R140/R172) (Figure 1B).

Only three of the ten (30%) *RUNX1*-mutated patients achieved a complete remission after intensive induction treatment, whereas the complete remission rate in the control group was 46/63 (73%; P=0.01). Four of ten (40%) *RUNX1*-mutated patients were primarily refractory to induction treatment, whereas this rate was only 11.5% in the control group (P=0.04).

The log-rank test identified *RUNX1* mutations as a significant strong negative predictor of relapse-free survival (P=0.002) and overall survival (P<0.001). The 3-year relapse-free and overall survival rates for *RUNX1* mutated patients were 0%, whereas they were 30.4% (relapse-free survival) and 34.4% (overall survival) for patients with wild-type *RUNX1*.

Kaplan-Meier estimates were calculated to display the negative prognostic influence of *RUNX1* mutations on overall survival in all 73 study patients and the ELN intermediate I and elderly subgroups of patients with AML (Figure 2).

In a multivariate model for overall survival, including age (10-year differences), the ELN genetic groups and *RUNX1* mutational status, all covariates were statistically significant parameters (Table 3).

Identification of genes differentially expressed between RUNX1*-mutated and* RUNX1*-wild type cases*

To gain insights into the biology of *RUNX1*-mutated CN-AML, we derived *RUNX1* mutation-associated gene expression signatures. Of note, *RUNX1* mutations were found exclusively in CN-AML patients with wild-type *NPM1*, while over 60% of *RUNX1*-wild type patients carried *NPM1* mutations which themselves are associated

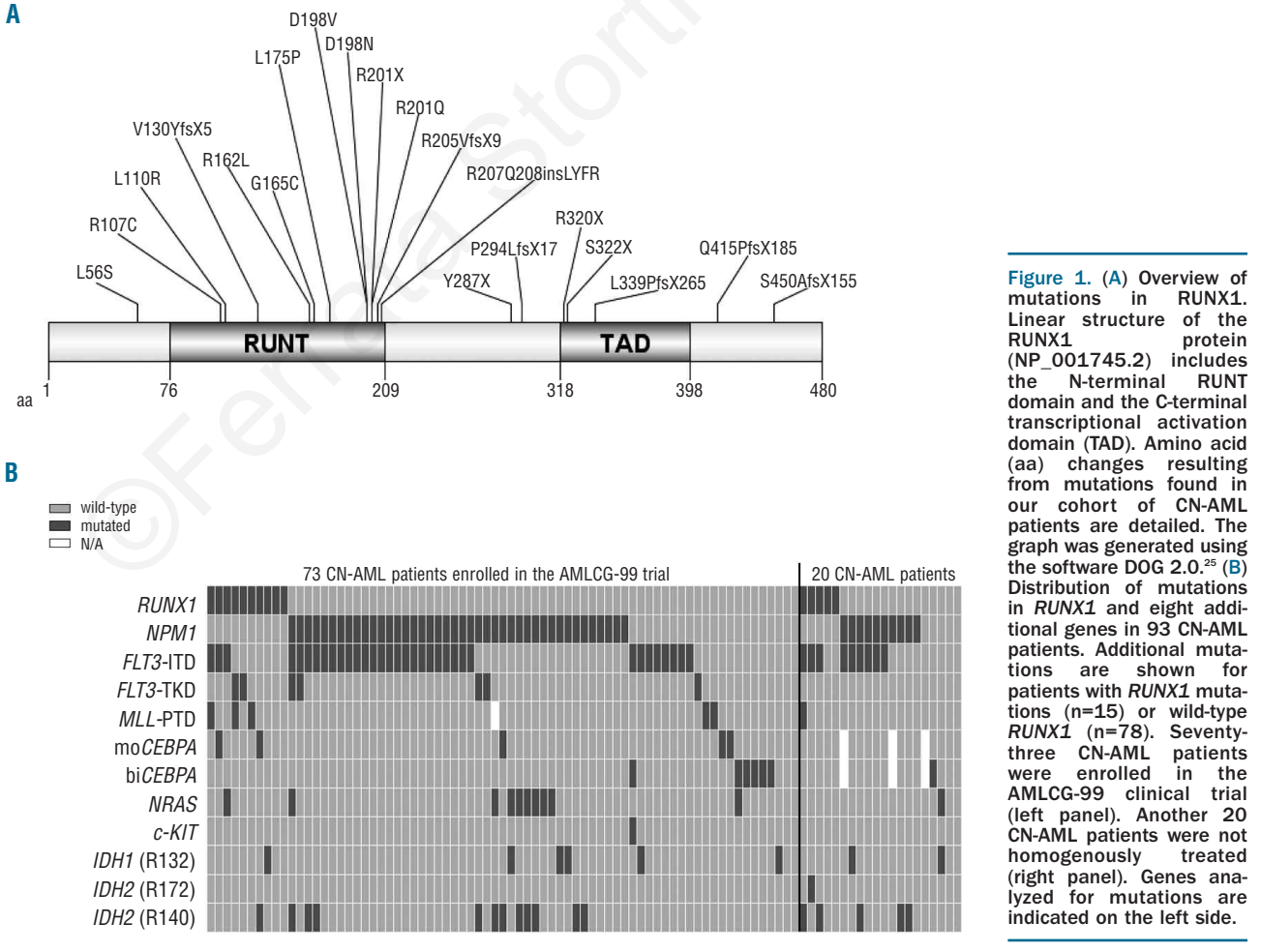

Figure 1. (A) Overview of mutations in RUNX1. Linear structure of the RUNX1 protein (NP_001745.2) includes the N-terminal RUNT domain and the C-terminal transcriptional activation domain (TAD). Amino acid (aa) changes resulting from mutations found in our cohort of CN-AML patients are detailed. The graph was generated using the software DOG 2.0.[25] (B) Distribution of mutations in *RUNX1* and eight additional genes in 93 CN-AML patients. Additional mutations are shown for patients with *RUNX1* mutations (n=15) or wild-type *RUNX1* (n=78). Seventy-three CN-AML patients were enrolled in the AMLCG-99 clinical trial (left panel). Another 20 CN-AML patients were not homogenously treated (right panel). Genes analyzed for mutations are indicated on the left side.

with a strong gene expression signature.[19] To avoid confounding our analyses through the impact of *NPM1* mutations, we only analyzed patients with wild-type *NPM1*. Comparing 15 *RUNX1*-mutated/*NPM1*-wild-type patients and 26 *RUNX1*-wild-type/*NPM1*-wild-type ones, we identified a set of 85 differentially expressed genes (Figure 3 and *Online Supplementary Table S2*). Sixty-nine genes showed higher expression in the *RUNX1*-mutated cases, while 16 genes were down-regulated. The most prominently up-regulated genes in *RUNX1*-mutated CN-AML include lymphoid regulators such as HOP homeobox (*HOPX*), deoxynucleotidyltransferase, terminal (*DNTT*) and B-cell linker (*BLNK*), indicating lineage infidelity.

To investigate whether specific functional pathways are over-represented among the genes deregulated in *RUNX1*-mutated CN-AML, we performed gene set enrichment analysis. We found that 71 gene sets were significantly enriched in the *RUNX1*-mutated patients, while 51 gene sets were enriched in the *RUNX1*-wild-type patients (*Online Supplementary Table S3*). Gene sets up-regulated in *RUNX1*-mutated patients included signaling pathways highly expressed in lymphoid cells, such as the B-cell receptor (*BCR*) signaling pathway and the toll-like receptor 4 (*TLR4*) and *NOTCH1* pathways (*Online Supplementary Figure S1 A-C*). Conversely, pathways related to DNA synthesis, DNA repair and DNA damage response pathways were down-regulated in *RUNX1*-mutated AML (*Online Supplementary Figure S1 D-F*).

Discussion

In our analysis of a homogeneous and uniformly treated cohort of CN-AML patients enrolled in the AMLCG-99 trial, we found *RUNX1* mutations in 13.7% of patients. This frequency is similar to that reported by Tang *et al.*[6] who found mutations in 13.9% of CN-AML, but higher than the frequency reported by Gaidzik *et al.* (3.9%)[5] who only studied patients below the age of 60 years. We confirmed that *RUNX1* mutations are more frequent in elderly, male patients and that these mutations are associated with some established genetic markers such as *MLL*-PTD (positively) and *NPM1* mutations (negatively).[5-7] In our cohort, *NPM1* and *RUNX1* mutations were mutually exclusive.

Our analyses revealed that *RUNX1* mutations are a highly significant predictor of inferior outcomes, including a lower complete remission rate, shorter relapse-free survival and shorter overall survival. A high proportion of patients with *RUNX1* mutations did not respond to intensive induction treatment, and only three out of ten (30%) achieved a complete remission. Even these three responders all died within 9.5 months (two in relapse; one in complete remission). *RUNX1* mutations were a significant covariate in a multivariate model for overall survival including age (≥60 years), the ELN genetic classification and *RUNX1* mutational status. These findings are consistent with those in a study by Tang *et al.*,[6] who reported

Table 1. Molecular details of *RUNX1* mutations in 94 CN-AML patients. Fifteen CN-AML patients carried *RUNX1* mutations. Four out of these 15 patients had several *RUNX1* mutations. Sequence variations in the cDNA and protein are indicated with reference to the longest isoform of *RUNX1* (NM_001754.4). UPN: Unique Patient Number.

cDNA (NM_001754.4)	Protein (NP_001745.2)	Exon	UPN	AMLCG-99
c.167T>C	p.(Leu56Ser)	4	1	Included
c.319C>T	p.(Arg107Cys)	4	2	-
c.329A>G	p.(Lys110Arg)	4	3	-
c.387_388insTATTG	p.(Val130Tyrfs*5)	5	4	-
c.485G>A	p.(Arg162Lys)	5	5	Included
c.493G>T	p.(Gly165Cys)	5	6	-
c.524T>C	p.(Leu175Pro)	6	7	Included
c.592G>A	p.(Asp198Asn)	6	3	-
c.593A>T	p.(Asp198Val)	6	8	-
c.601C>T	p.(Arg201*)	6	9	Included
c.602G>A	p.(Arg201Gln)	6	8	Included
c.611_612insTGTCCCACAGGGAAAAGCTTCAC TCTGACCATCACTGTCTTCACAAACCCACCGC AAGTCGCCACCTACCACAGAGCCATCAAAAT	p.(Arg205Valfs*9)	6	8	-
c.620_621insACTTTACTTCCG	p.(Arg207_Gln208insLeuTyrPheArg)	7	3	-
c.861C>G	p.(Tyr287*)	8	10	Included
c.881delC	p.(Pro294Leufs*17)	8	11	Included
c.958C>T	p.(Arg320*)	8	4	-
c.965C>G	p.(Ser322*)	8	9	-
c.1003_1015dupCAGTTCCCCGCGC	p.(Leu339Profs*265)	9	12	Included
c.1243dup	p.(Gln415Profs*185)	9	13	Included
c.1243dup	p.(Gln415Profs*185)	9	14	-
c.1347_1348insGCTTCCTTCCTCCTAG	p.(Ser450Alafs*155)	9	15	Included

that *RUNX1* mutations are associated with shorter relapse-free survival and overall survival in homogeneously treated CN-AML patients. In contrast, Gaidzik *et al.* previously reported a negative prognostic impact of *RUNX1* mutations in a cytogenetically heterogeneous cohort, but found no significant impact on relapse-free or overall survival within the CN-AML subset.[5] Of note, their study only included younger patients, suggesting that the negative impact of mutated *RUNX1* might be age-related. Our findings strongly suggest that CN-AML patients with *RUNX1* mutations do not benefit from standard treatment. Screening for *RUNX1* mutations might, therefore, identify candidates for alternative treatment approaches. In summary, *RUNX1* mutational status might be considered for inclusion in a revised version of the ELN AML risk classification, particularly for older patients.

In addition, we demonstrated that patients with *RUNX1*-mutated CN-AML have a distinct gene expression pattern characterized by differential expression of 85

Table 2. Patients' characteristics. Correlation of clinical characteristics and *RUNX1* mutation status is indicated for 73 patients enrolled in the AMLCG-99.

Variable	wild-type *RUNX1*	Mutated *RUNX1*	*P* value
N. of patients	63	10	
Median age, years (range)	54 (27-83)	73 (54-78)	**0.001**
Male sex, n. (%)	26 (41.3)	9 (90)	**0.005**
White-cell count, x10⁹/L, median(range)	39.5 (0.1-486.0)	11.70 (1.8-105.3)	0.08
Hemoglobin, g/dL, median(range)	9.7 (5.5-14.2)	8.4 (4.9-9.3)	0.76
Platelet count, x10⁹/L,median(range)	52.0 (0.02-268.0)	37.0 (18.0-111.0)	0.52
LDH (U/L), median(range)	694 (181-2814)	328 (186-784)	**0.003**
Bone marrow blasts, %, median(range)	80 (20-100)	85 (20-95)	0.82
Performance Status (ECOG) ≥ 2 (%)	21 (35)	5 (50)	0.48
De novo AML (%)	57 (93.4)	8 (80)	0.2
French-American-British classification			
M0, n. (%)	1 (1.6)	1 (10)	0.26
M1, n. (%)	11 (18)	6 (60)	**0.01**
M2, n. (%)	20 (32.8)	2 (20)	0.71
M4, n. (%)	19 (31.1)	1 (10)	0.26
M5, n. (%)	9 (14.8)	0 (0)	0.34
M6, n. (%)	1 (1.6)	0 (0)	1
NPM1 mutated, n. (%)	42 (66.7)	0 (0)	**<0.001**
FLT3-ITD, n. (%)	31 (49.2)	3 (30)	0.32
FLT3-TKD	5 (7.9)	2 (20)	0.24
Monoallelic CEBPA mutated, n. (%)	3 (4.8)	2 (20)	0.14
Biallelic CEBPA mutated, n. (%)	6 (9.5)	0 (0)	0.59
MLL-PTD, n. (%)	2 (3.2)	3 (30)	**0.02**
NRAS mutated, n. (%)	9 (14.3)	1 (10)	1
KIT mutated, n. (%)	1 (1.6)	0 (0)	1
IDH1 R132 mutated., n. (%)	5 (7.9)	1 (10)	1
IDH2 R140 mutated, n. (%)	11 (17.5)	1 (10)	1
IDH2 R172 mutated, n. (%)	0	0	
ELN classification			
Favorable (ELN I), n. (%)	27 (42.9)	2 (20)	0.3
Complete remission, n. (%)	46 (73)	3 (30)	**0.01**
Non-responder AML, n. (%)	7 (11.5)	4 (40)	**0.04**

LDH: lactate dehydrogenase; ECOG: Eastern Cooperative Oncology Group.

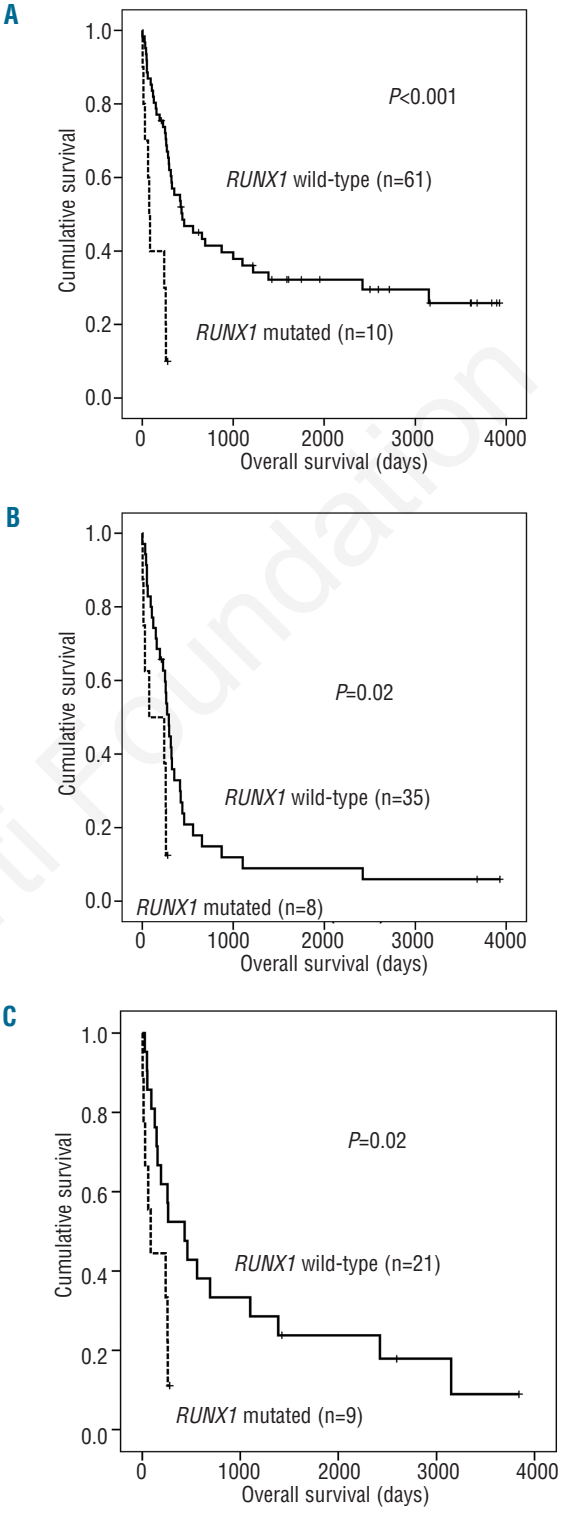

Figure 2. Influence of *RUNX1* mutations on clinical outcome. Kaplan-Meier estimates for intensively treated CN-AML patients with or without *RUNX1* mutations. The censored patient in the *RUNX1* mutated group experienced a relapse and was then lost to follow up. (A) The median overall survival in *RUNX1* mutated patients was 75 days compared to 442 days for patients with *RUNX1* wild-type status. (B) For ELN Intermediate I patients (CN-AML with wild-type *CEBPA* and wild-type *NPM1* and/or *FLT3*-ITD) median overall survival in *RUNX1* mutated patients was 75 days compared to 293 days for patients without this mutation. (C) In elderly AML patients (≥ 60 years) the median overall survival for *RUNX1*-mutated patients was 86 days and 432 days for patients with wild-type *RUNX1*.

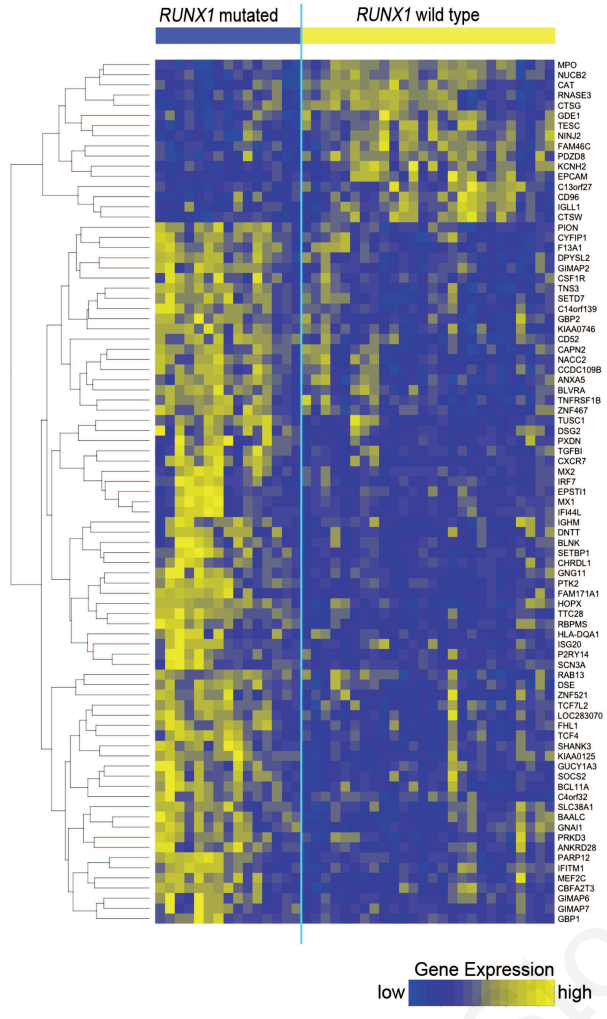

Figure 3. **Heatmap of genes differentially expressed between *RUNX1*-mutated and *RUNX1*-wild-type patients. Each column represents one of 41 CN-AML patients, grouped according to *RUNX1* mutation status, and each row represents one of 85 genes that were differentially expressed. Yellow indicates high and blue indicates low gene expression.**

genes. Twenty-six out of these 85 differentially expressed genes were previously reported to be deregulated in *RUNX1* mutated AML M0 (minimally differentiated AML according to the French-American-British classification), indicating that the expression of these genes is very likely to be influenced by *RUNX1* mutations.[8] These 26 genes include the T-cell markers *DNTT* and *BLNK*, suggesting that mutations in the early hematopoietic stem cell regulator *RUNX1* may disturb differentiation resulting in lineage infidelity in early progenitor cells. Since AML M0 is cytogenetically diverse, the study by Silva *et al.* is limited by the influence of multiple cytogenetic aberrations including complex karyotype and trisomy 13.[8,20]

Table 3. **Multivariate Cox regression model with covariates *RUNX1* mutational status, age (10-year difference) and the European LeukemiaNet AML risk classification (ELN).**

Variable	Overall survival[1]	
	HR (95% CI)	*P* value
RUNX1 mutation	2.51 (1.1-5.8)	0.03
Age[2]	1.24 (1.01-1.52)	0.04
ELN	4.35 (2.19-8.63)	<0.001

[1]N=71/73 (97.3%); two observations missing because of missing follow-up data; [2]The hazard ratio (HR) refers to a 10-year difference in age. CI: confidence interval.

Furthermore, Silva *et al.* limited their mutation screening to the RUNT-domain of *RUNX1*, which likely resulted in an underestimation of the *RUNX1* mutation burden. In contrast, our study of *RUNX1* mutations in CN-AML is not biased by the impact of cytogenetic aberrations on gene expression and an underestimation of *RUNX1* mutations (the complete coding sequence of *RUNX1* gene was sequenced). Gaidzik *et al.* also studied the association between *RUNX1* mutational status and gene expression in a large cohort of AML patients including various cytogenetic subgroups.[5] However, that cohort included only seven CN-AML patients with *RUNX1* mutations and several different microarray platforms were used in their study, thus limiting the comparability with the study presented here.

High levels of expression of several genes that we found up-regulated in *RUNX1*-mutated CN-AML, namely *DNTT*, *SETBP1*, *BAALC* and *PTK2*, had previously been shown to be associated with adverse prognosis in AML.[21-24]

In summary, we provide further evidence for the unfavorable impact of *RUNX1* mutations on clinical outcomes in a cytogenetically homogeneous and uniformly treated cohort of AML patients. Compared to previous studies on *RUNX1* mutation-related gene expression signatures which were based on cytogenetically diverse cohorts of patients,[5,8] our study specifically focused on CN-AML. Our findings reveal the unique biology of *RUNX1*-mutation-positive AML and may provide the basis for the development of novel diagnostic tools and therapies. Importantly, our findings that *RUNX1* mutations in elderly CN-AML patients are associated with a dismal prognosis should aid in defining these patients as a group that could potentially benefit from alternative treatment strategies.

Authorship and Disclosures

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